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Recent insights into zebrafish cardiac regeneration

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In humans, myocardial infarction results in ventricular remodeling, progressing ultimately to cardiac failure, one of the leading causes of death worldwide. In contrast to the adult mammalian heart, the zebrafish model organism has a remarkable regenerative capacity, offering the possibility to research the bases of natural regeneration. Here, we summarize recent insights into the cellular and molecular mechanisms that govern cardiac regeneration in the zebrafish.

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Introduction

Cardiovascular disease remains a dominant cause of death worldwide and the burden of cardiomyopathies is predicted to increase substantially in the future [1]. Myocardial infarction results from the formation of atherosclerotic plaques and the blockage of coronary arteries, which fail to deliver nutrients and oxygen to the myocardium, causing the death of millions of cardiac cells. The replacement of the damaged tissue by non-contractile scar tissue protects the heart from wall rupture but ultimately leads to pathologies such as adverse cardiac remodeling and heart failure (reviewed in Ref. [2]).

For decades, the adult mammalian myocardium was considered a post-mitotic tissue with very little to no regenerative capacity [3]. Postnatal cardiac growth is predominantly a result of cardiomyocyte hypertrophy mediated by additional DNA synthesis without cytokinesis, generating mononuclear polyploid and binucleated diploid cardiomyocytes in humans and mouse, respectively [4]. Remarkably, the neonatal mouse heart is able to regenerate during a short period after birth [5]. Of interest, a case reported complete functional recovery after severe myocardial infarction in a human newborn [6]. This observation might suggest that the transient cardiac regenerative capacity in neonatal mice is conserved, at least partially, in humans, and that a latent regenerative capacity is actively suppressed during maturation [7]. Accordingly, the exploration of how other species retain cardiac regenerative capacity throughout their lifespan continues to garner interest.

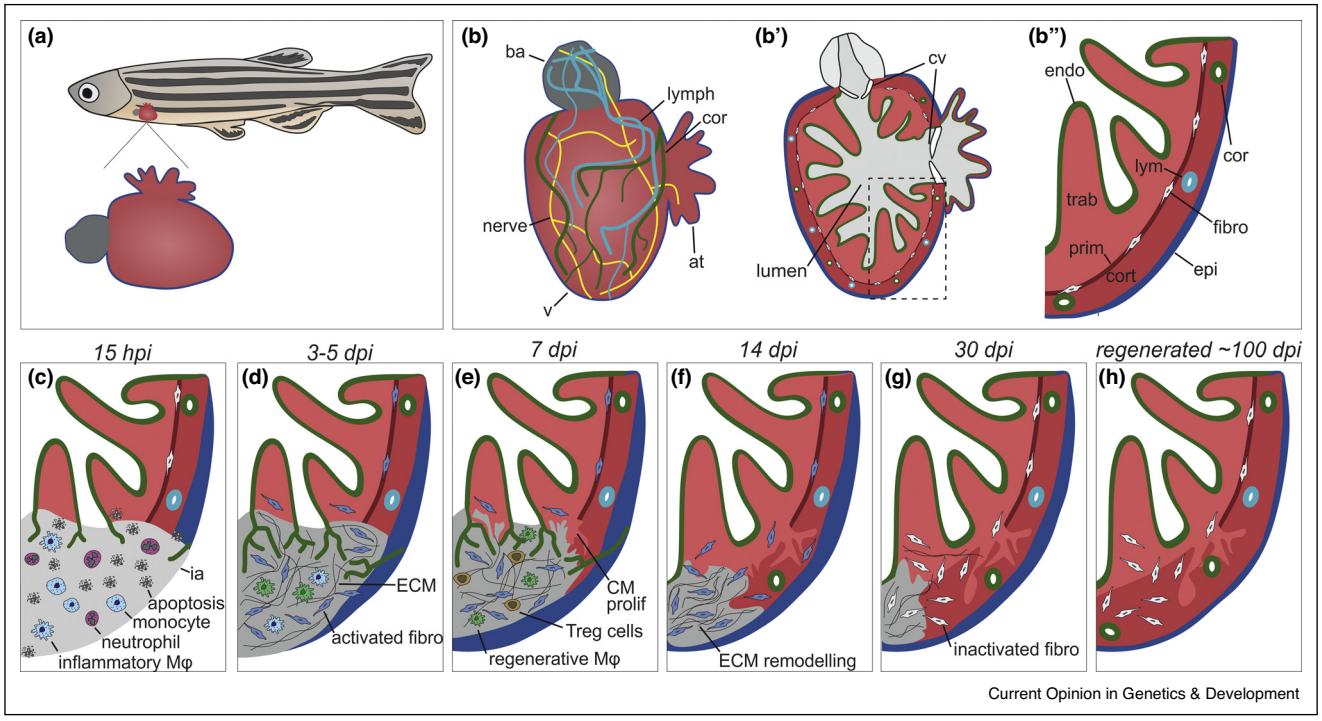
The zebrafish (*Danio rerio*) is one of the most relevant models to study regenerative biology given its fascinating capacity to regenerate most of its organs and tissues, including the heart (reviewed in Ref. [8]). The biological response to cardiac injury in the zebrafish requires the orchestrated participation of multiple cell types involving numerous molecular mechanisms that ultimately result in the regeneration of the damaged tissue. Here, we summarize recent discoveries on cardiac regeneration in the adult zebrafish that provide mechanistic insights into how this complex process is successfully achieved.

Cardiac regeneration in the zebrafish

The zebrafish heart shares numerous similarities with its mammalian equivalent with regards to morphology, cellular composition, genetic regulation and also embryonic development (reviewed in Ref. [9]). During development, cardiac progenitors derived from the first heart field initially form a primordial heart tube. This structure elongates and loops to form a two chambered embryonic heart by the incorporation of cardiac progenitors from the second heart field to the venous and arterial poles [10–13]. The adult cardiac muscle, or myocardium, is lined by an endocardial layer facing the lumen and covered by an epicardial layer. The zebrafish heart is two-chambered, with the single atrium and ventricle connected by an atrio-ventricular valve. Blood enters the heart through the atrium, is pumped by the ventricle and is ejected into the circulation through the bulbus arteriosus, a prominent outflow tract. The myocardium can be subdivided into three main layers: the inner trabecular layer, the primordial layer and the outer cortical layer (Figure 1a–b″).

A seminal study by Poss and colleagues showed that upon resection of 20% of the adult zebrafish ventricle, the lost myocardium is replaced by newly functional cardiac muscle, achieving regeneration by a virtually scar-free process [14]. Later, further cardiac injury models were developed, including ventricular cryoinjury and genetic ablation. The ventricular cryoinjury induces cell death by fast freezing part of the ventricle [15–17]. Cryoinjured hearts are also able to regenerate, but regeneration occurs concomitant with the transient deposition of a fibrotic

Figure 1



Representation of cardiac regeneration in the adult zebrafish.
(a) Adult zebrafish heart anatomical position. **(b)** Overview of the uninjured zebrafish heart, comprising the atrium, ventricle and bulbus arteriosus. The heart is covered and wired by the epicardium, lymphatic system, coronary arteries and nerves. **(b′)** Section of the zebrafish heart. Cardiac valves separate the chambers. **(b″)** Zoomed region of (b′). Three myocardial layers can be identified: trabecular, primordial, and cortical myocardium. The endocardium coats the lumen. The cortical layer is covered by the epicardium. Fibroblasts lie between the cortical and trabecular myocardium. **(c)–(h)** Timeline of cardiac regeneration events upon cryoinjury. (c) Fast freezing of the ventricular apex leads to the formation of the injury area. Necrotic and apoptotic cells trigger an inflammatory response characterized by the infiltration and activation of neutrophils, monocytes, and macrophages, among others. Endothelial and epicardial cells are activated and infiltrate the injury area. (d) The acute inflammation regresses and activated fibroblasts elicit a fibrotic response by depositing extracellular matrix (ECM). (e) Peak of cardiomyocyte proliferation followed by migration along epicardial and endocardial cells. T_{reg} cells home to the injured tissue. (f) The ECM remodels and cardiomyocyte proliferation continues. (g) Fibroblasts undergo inactivation and the fibrotic scar regresses. (h) Complete regression of the fibrotic scar and replenishment by functional myocardium. The cortical myocardial layer remains thickened and the primordial layer does not regenerate. Abbreviations: at, atrium; ba, bulbus arteriosus; CM prolif, cardiomyocyte proliferation; cor, coronary arteries; cv, cardiac valves; ECM, extracellular matrix; epi, epicardium; endo, cardiac endothelium; dpi, days post injury; fibro, fibroblast; ia, injury area; hpi, hours post injury; lymph, the lymphatic system; Mφ, macrophage; prim, primordial layer; trab, trabecular layer; v, ventricle.

scar, which is ultimately resolved [15] (Figure 1c–h). The third main injury model, genetic ablation of cardiomyocytes, is currently based on the inducible and tissue-specific expression of either diphtheria toxin A [18] or nitroreductase, an enzyme that converts the prodrug metronidazole into a cytotoxic metabolite that induces cell death [19]. These methods, and others, have been used extensively to interrogate cardiac regenerative mechanisms in the zebrafish.

Cellular source of the regenerated myocardium

Regarding heart regeneration, one central question to be resolved is: where do new cardiomyocytes come from? The current consensus is that newly formed cardiomyocytes derive from preexistent differentiated cardiomyocytes (Figure 1e). This hypothesis is strongly supported by lineage tracing studies using the Cre-lox technology, in which the cardiomyocytes from uninjured hearts were irreversibly tagged using the cardiomyocyte-specific promoter *cmlc2* (*myl7*) [20,21]. Of note, *myl7* starts to be expressed in cardiomyocyte progenitor cells within the anterior lateral mesoderm before cardiac looping [22]. Thus, not only fully differentiated cardiomyocytes express *myl7*, a fact to consider when interpreting *myl7* fate mapping studies during adult heart regeneration. In response to injury, some cardiomyocytes, predominantly those located in subepicardial regions and close to the injury border, reactivate the expression of regulatory regions of *gata4* [20] and *ctgfa* [23•] genes. More recently, the expression of

sox10, a well-known neural crest marker, was shown to label a subset of cardiomyocytes in the embryonic [24] and adult [25•] zebrafish heart. These cells proliferate preferentially and contribute to the regenerated myocardium following cardiac injury [26•,27•]. These findings might represent a contribution of neural crest-derived cardiomyocytes to cardiac regeneration or, alternatively, the activation of specific neural crest genetic signatures within some proliferating cardiomyocytes. In sum, the extent to which some cardiomyocytes present a high regenerative capacity, and which specific cellular and transcriptomic changes are involved in this process, warrants further investigation.

Continuing this theme, there is evidence that cardiomyocytes can partially switch their fate during regeneration and rebuild different myocardial layers. For example, ablation of embryonic ventricular cardiomyocytes can be compensated by atrial cardiomyocytes [28]. Furthermore, clonal analysis in resected ventricles suggested that cortical cardiomyocytes contribute to the regenerated cortical layer, indicating a commitment to a particular myocardial compartment [29]. More recently, trabecular cardiomyocytes have been shown to also regenerate the cortical layer, which reveals some degree of cardiomyocyte plasticity [30•] (Figure 1h). Whether cortical cardiomyocytes can contribute to the regenerated trabeculae is currently unknown. Interestingly, the primordial layer of the myocardium is not regenerated in cryoinjured hearts [23•] (Figure 1h). This observation, together with the discovery that the regenerated cortical layer remains thickened in resected and cryoinjured hearts [14,15], (Figure 1h) and that ventricular wall contractility is not completely reestablished [31], indicates that myocardial regeneration is not fully achieved in the zebrafish.

The finding that adult cardiomyocytes in the zebrafish are predominantly diploid [32] has long been regarded as a possible explanation for their high proliferative potential. Cardiomyocyte polyploidy is more frequent in non-regenerative than in regenerative species and represents a barrier to proliferation [33,34]. Indeed, polyploidization of cardiomyocytes is associated with the loss of cardiac regenerative and reparative capacity in mice [5,35]. Notably, elegant genetic models have revealed that an increase in cardiomyocyte ploidy reduces cardiac regenerative capacity in zebrafish, pointing to a pivotal role for ploidy in this process [36••].

An important quest is the identification of endogenous and exogenous molecules and environmental stimuli inducing cardiomyocyte proliferation. The tyrosine-protein kinase receptor Erbb2 is one of the main mediators of cardiomyogenesis during regeneration. One of its ligands, Neuregulin 1 (Nrg1), is a potent cardiomyocyte mitogen sharply induced in perivascular cells during cardiac regeneration [37]. Erbb2 signaling also acts downstream participates of the effector cascade of vitamin D [38•] or hemodynamic forces [39] during cardiomyocyte proliferation. Erbb2 signaling mediates a switch from oxidative phosphorylation to a glycolysis predominant metabolism observed in proliferating cardiomyocytes [40•]. Interestingly, Erbb2 signaling has also been clearly associated to heart regeneration in the neonatal mouse [41]. Additional signaling pathways that influence cardiomyocyte proliferation have been identified, including PPARδ [42] and *vegfaa* [43]. Whether these also interact with Erbb2 signaling pathway is not known.

Extensive epigenetic remodeling precedes a regenerative response in cardiomyocytes. The repression of sarcomeric and cytoskeletal genes by H3K27me3-mediated epigenetic silencing is a pre-requisite for cell cycle re-entry [44•]. Specific enhancers become activated during injury response, as explored by histone H3.3 profiling [45]. Furthermore, transient cell membrane fusions in cardiomyocytes [46] have been shown to play a role in myocardial regeneration. Additional factors acting at the organismal level also influence cardiomyocyte proliferation including swimming-induced exercise [47] and cardiac preconditioning [48].

Overall, a tight temporal and spatial control of mitogenic signals is crucial to promote cardiomyocyte proliferation and heart regeneration. The coordinated participation of other cell types, however, is necessary to successfully achieve this complex process.

Immune system response

Following cardiac injury, there is an initial pro-inflammatory phase in which necrotic cells trigger the activation and infiltration of immune cells. These cells, both from intra-cardiac and extra-cardiac origin, clear debris and dead cells and remodels the extracellular matrix (ECM) (Figure 1c–e). Several immune cell types participate in this process in a timely and spatially coordinated manner (reviewed in Ref. [49]). For example, increased neutrophil retention [50,51] or ablation of T_{reg} cells [52] lead to reduced organ regenerative capacity.

In mammals, cardiac-resident macrophages are the most abundant immune cell populations in the heart and the majority of them are derived from the yolk sac [53]. Depletion of macrophages leads to impaired heart regeneration in neonatal mice [54] and zebrafish [55••]. Remarkably, a comparative analysis between zebrafish and medaka (*Oryzias latipes*), also a teleost but unable to regenerate the heart [56], revealed substantial differences in the immune response upon cardiac injury [55••]. For instance, the stimulation of the Toll-like receptor in medaka promoted immune cell recruitment, neovascularization, neutrophil clearance, cardiomyocyte proliferation and scar resolution. Alternatively, delayed macrophage recruitment in zebrafish results in compromised

neovascularization, neutrophil clearance, cardiomyocyte proliferation and scar resolution [55••].

The role of macrophages has been further defined by the identification of pro-inflammatory macrophages expressing *tumor necrosis factor a* (*tnfa)* at early stages upon cardiac insult [57•], in line with what was previously reported during embryonic caudal fin regeneration [58]. Furthermore, pro-regenerative macrophages expressing *wilms tumor 1b (wt1b)* show specific recruitment dynamics and genetic signatures during heart regeneration [59•]. Moreover, *osteopontin*-positive macrophages are implicated in triggering a fibrotic response as well as fibrosis regression [57•]. Overall, a finely tuned temporal and spatial control of inflammation is crucial for heart regeneration. Yet, the identification of additional immune cell types and specific subpopulations involved in cardiac regeneration in the zebrafish remains to be fully explored.

Cardiac endothelium, nerves and lymphatic system

The cardiac endothelium is composed by two structures: the coronary and the endocardial endothelium [60]. Angiogenic sprouting infiltrating the damaged tissue is observed as early as 15 hours post injury (Figure 1c). Inhibition of this process by overexpression of a *vegfaa* dominant-negative isoform diminishes cardiomyocyte proliferation and abrogates cardiac regeneration [61]. The peak of proliferation of endocardial cells surrounding the damaged tissue occurs between 3 and 5 dpi, before the cardiomyocyte proliferation peak rate at 7 dpi (Figure 1d,e). In this context, the participation of Notch [62] and Wnt [63] signaling in endocardial cells has been described. Beyond their function in oxygenation and nutrient delivery, regenerating coronaries serve as a scaffold for cardiomyocytes to repopulate the injured area, with the epicardial Cxcl12/Cxcr4 signaling axis playing an important role in this process [64•].

Cardiac innervation also influences the regenerative process. Hypo-innervation of adult zebrafish heart leads to reduced cardiomyocyte proliferative potential, abrogating cardiac regeneration [65]. While the role of the lymphatic system has long remained enigmatic in the regenerative context, recent studies indicate its importance in fluid drainage and inflammatory cells removal from the damaged myocardium [66•,67•,68•].

Overall, these results establish an essential role for the endocardium, coronary endothelium, nerves and lymphatic system to support and promote cardiac regeneration as a source of signals but also as a physical scaffold.

Fibrotic scar origin and fate

During cardiac regeneration, the epicardium and epicardium-derived cells (EPDCs) contribute to the generation of perivascular cells and fibroblasts, which are important for scar deposition and remodeling [69,70••]. Indeed, genetic ablation of *tcf21*$^{+}$ epicardial cells reduces the proliferative capacity of cardiomyocytes [71]. Collective migration of epicardial cells is reliant on the generation of polyploid epicardial leader cells at the migration front [72]. Interestingly, epicardial cells secrete the ECM substrates needed for their migration over the cardiac surface [73]. The epicardium has also been suggested to secrete trophic factors important for heart regeneration, including mitogenic signals such as neuregulin 1 [37]. In addition, EPDCs crosstalk with other cell types, mediated for example by Neuropilin 1, a transmembrane receptor whose ligands include platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), which mediates epicardial activation and revascularization during regeneration [74].

Fibroblasts are the main source of collagen and other ECM-proteins upon cardiac injury. The inactivation of pre-existing cardiac fibroblasts, partly derived from the embryonic epicardium, occurs during the scar resolution phase [70••] (Figure 1g,h). Moreover, cellular senescence is observed at the injury site in the zebrafish and a correct balance of senescent cells might be necessary for heart regeneration [75,76]. Studies in neonatal mice showed that fibroblast senescence is required for cardiac regeneration [76,77], and this needs to be confirmed in the zebrafish model. Remarkably, genetic ablation of collagen-producing cells upon heart injury is detrimental for cardiomyocyte proliferation in the zebrafish [70••]. The composition and stiffness of the zebrafish cardiac ECM is dynamic in composition and stiffness during injury resolution [78] (Figure 1d–g). Yet, much remains to be learned regarding which specific signals, components, or physicochemical properties of zebrafish ECM influence heart regeneration.

Outlook and future perspectives

The last few years have yielded significant breakthroughs in our understanding of the different cell types and cell interactions influencing myocardial regeneration in the zebrafish. We gained an improved perspective on how the different cardiac structures contribute to heart regeneration. We also learned that several cellular and molecular mechanisms are conserved between zebrafish and neonatal mouse regeneration. Furthermore, the zebrafish has also proven to be an excellent model to study cardiac valve regeneration [79•,80•]. These findings represent an important added value to the model, given that numerous degenerative and congenital diseases known to affect cardiac valves are important health concerns. With the rapid development of omićs-based approaches, databases integrating available information – for example, [81] – will be of immense benefit to the community. The functional validation of how transcriptome and cellular changes are integrated within different cell types and how the

outcome influences cardiac regeneration will become one of the next big challenges in the field. In this regard, the continued establishment of efficient technologies for tissue-specific and cell type-specific genetic manipulations will be ever more relevant. Finally, perfoming cross-species analysis to define which results have a translational value will be important future steps towards unravelling the complicated processes of heart regeneration.

Conflict of interest statement

Nothing declared.

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